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(54) Title: **NEW METHODS FOR DIAGNOSIS AND TREATMENT OF TUMOURS**

(57) Abstract: The present invention relates to new methods for diagnosis and treatment of tumours, using novel peptides for binding radionuclides.

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New methods for diagnosis and treatment of tumours

Description

5

Field of the invention

The present invention relates to new methods for diagnosis and treatment of tumours, using novel peptides for binding radionuclides.

10

Brief description of the background art

Tumours cannot gain more than a certain weight without the formation of new blood vessels (angiogenesis), and a correlation between microvessel density and tumour invasiveness has been reported for a number of tumours (Folkman (1995), *Nature Med.*, 1, 27 – 31). Moreover, angiogenesis is involved in the majority of ocular disorders which result in loss of vision (Lee et al., *Surv. Ophthalmol.* 43, 245 – 269 (1998); Friedlander, M. et al., *Proc. Natl. Acad. Sci. U.S.A.* 93, 9764 – 9769 (1996)). Molecules capable of selectively targeting markers of angiogenesis would create clinical opportunities for the diagnosis and therapy of tumours and other diseases characterised by vascular proliferation, such as diabetic retinopathy and age-related macular degeneration. Markers of angiogenesis are expressed in the majority of aggressive solid tumours in association with tumoural vessels and should therefore be readily accessible to specific binders injected intravenously (Pasqualini et al., (1997), *Nature Biotechnol.*, 15, 542 – 546; Neri et al. (1997), *Nature Biotechnol.*, 15, 1271 – 1275). Targeted occlusion of the neovasculature may result in tumour infarction and collapse (O'Reilly et al. (1996), *Nature Med.*, 2, 689 – 692; Huang et al. (1997), *Science*, 275, 547 – 550).

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The ED-B domain of fibronectin, a sequence of 91 amino acids identical in mouse, rat and human, which is inserted by alternative splicing into the fibronectin molecule, specifically accumulates around neo-vascular structures (Castellani et al. (1994), Int. J. Cancer 59, 612 – 618) and could represent a target for molecular intervention. Indeed, it has recently been shown with fluorescent techniques that anti-ED-B single-chain Fv antibody fragments (scFv) accumulate selectively around tumoural blood vessels of tumour-bearing mice, and that antibody affinity appears to dictate targeting performance (Neri et al. (1997), Nature Biotechnol., 15, 1271 – 1275; WO 97/45544).

Furthermore, antibodies and antibody fragments specific for binding the ED-B domain of fibronectin with a sub-nanomolar dissociation constant as well as radiolabeled derivatives thereof are described in WO 99/58570. The biodistribution of one of these high-affinity human antibody fragments, the ¹²⁵I labelled antibody fragment called L19, was already investigated in tumour-bearing mice (Tarli et al., Blood, Vol. 94, No. 1 (1999), p. 192 – 198). Radiolabeled conjugates comprising L19-antibodies and their use for the detection and treatment of angiogenesis are disclosed in WO 01/62800.

The recombinant production of functionalized single-chain Fv antibody fragments binding to the ED-B domain of the B-isoform of fibronectin in *Pichia pastoris* has already been described (Marty et al., Protein Expression and Purification 21, 156 – 164 (2001)).

Further, radiolabeling of scFv antibody fragments with ^{99m}Tc through a C-terminal cysteinyl peptide was described by George et al., Proc. Natl. Acad. Sci. USA, Vol. 92 pp. 8358 – 8362, 1995, and by Verhaar et al., J. Nuc. Med., Vol. 37(5), pp. 868 – 872, 1996.

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However, there is still a clinical need for providing antibody fragments that have improved pharmacokinetic properties, and that can easily be labeled with radioisotopes of e.g. Technetium or Rhenium, since these radionuclides are particular well suited for radiopharmaceuticals.

5

Object of the invention

It is therefore an object of the invention to provide antibody fragments that have improved pharmacokinetic properties, particularly target specificity
10 and/or in vivo stability, and that can easily bind radioisotopes e.g. of Technetium or Rhenium.

Summary of the invention

15 The present invention describes compounds comprising a peptide comprising

20 aa) the sequence of the antigen-binding site for the extra domain B (ED-B) of fibronectin comprising complementarity-determining regions HCDR3 and/or LCDR3 as shown in Table 1 or a variation thereof that is a deletion, insertion and/or substitution of up to 5 amino acids for the HCDR3 region and up to 6 amino acids for the LCDR3 region which has the same function as a peptide according to Seq. Id. No. 1;

25

30 ab) the sequence of the antigen-binding site for the extra domain B(ED-B) of fibronectin comprising complementarity-determining regions HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 as shown in Table 1 or a variation thereof that is a deletion, insertion and/or substitution of up to 3 amino acids for the HCDR1 region, up to 8 amino acids for the HCDR2 region, up to 5 amino acids for the HCDR3 region, up to 6

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amino acids for the LCDR1 region, up to 4 amino acids for the LCDR2 region and up to 6 amino acids for the LCDR3 region; which has the same function as a peptide according to Seq. Id. No. 1;

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- ac) the sequence according to Seq. Id. No. 1 (L19) or a variation of Seq. Id. No. 1 that is a deletion, insertion and/or substitution of up to 30 amino acids, and which has the same function as a peptide according to Seq. Id. No. 1,

10

and

15

- ba) an amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys (Seq. Id. No. 2), wherein Xaa₁, Xaa₂, and Xaa₃ each independently represent any naturally occurring amino acid or

20

- bb) an amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys-Xaa₄ (Seq. Id. No. 3), wherein Xaa₁, Xaa₂, Xaa₃, and Xaa₄ each independently represent any naturally occurring amino acid or

25

- bc) an amino acid sequence (His)_n (Seq. Id. No. 4), wherein n stands for an integer from 4 to 6,

wherein the C-terminus of aa), ab) or ac) is bound to the N-terminus of one of the sequences Seq. Id. No. 2, Seq. Id. No. 3 or Seq. Id. No. 4 via a peptide bond.

30

The compounds are preferably single chain antibody fragments, particularly scFv fragments. Further, the compounds are preferably conjugated to a radioisotope, e.g. a radioisotope of Technetium, such as ^{94m}Tc, ^{99m}Tc Rhenium, such as ¹⁸⁶Re, ¹⁸⁸Re, or other isotopes, such as ²⁰³Pb, ⁶⁷Ga, ⁶⁸Ga,

$$^{43}\text{Sc}, ^{44}\text{Sc}, ^{47}\text{Sc}, ^{110\text{m}}\text{In}, ^{111}\text{In}, ^{97}\text{Ru}, ^{62}\text{Cu}, ^{64}\text{Cu}, ^{67}\text{Cu}, ^{68}\text{Cu}, ^{86}\text{Y}, ^{88}\text{Y}, ^{90}\text{Y},$$

$$^{121}\text{Sn}, ^{161}\text{Tb}, ^{153}\text{Sm}, ^{166}\text{Ho}, ^{105}\text{Rh}, ^{177}\text{Lu}, ^{72}\text{As} \text{ and } ^{18}\text{F}.$$

The present invention also describes a pharmaceutical composition
5 comprising the above compound as active agent together with
physiologically acceptable adjuvants, diluents and/or carriers.

The present invention also describes the use of a peptide comprising

- 10 aa) the sequence of the antigen-binding site for the extra domain
 B (ED-B) of fibronectin comprising complementarity-
 determining regions HCDR3 and/or LCDR3 as shown in Table
 1 or a variation thereof that is a deletion, insertion and/or
15 substitution of up to 5 amino acids for the HCDR3 region and
 up to 6 amino acids for the LCDR3 region which has the same
 function as a peptide according to SEQ Id. No. 1;
- 20 ab) the sequence of the antigen-binding site for the extra domain
 B(ED-B) of fibronectin comprising complementarity-
 determining regions HCDR1, HCDR2, HCDR3, LCDR1, LCDR2
 and LCDR3 as shown in Table 1 or a variation thereof that is
 a deletion, insertion and/or substitution of up to 3 amino acids
 for the HCDR1 region, up to 8 amino acids for the HCDR2
 region, up to 5 amino acids for the HCDR3 region, up to 6
25 amino acids for the LCDR1 region, up to 4 amino acids for the
 LCDR2 region and up to 6 amino acids for the LCDR3 region;
 which has the same function as a peptide according to SEQ
 Id. No. 1;
- 30 ac) a sequence according to Seq. Id. No. 1 (L19) or a variation of
 Seq. Id. No. 1 that is a deletion, insertion and/or substitution

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of up to 30 amino acids, and which has the same function as a peptide according to Seq. Id. No. 1,

and

5

ba) an amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys (Seq. Id. No. 2), wherein Xaa₁, Xaa₂, and Xaa₃ each independently represent any naturally occurring amino acid or

10

bb) an amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys-Xaa₄ (Seq. Id. No. 3), wherein Xaa₁, Xaa₂, Xaa₃, and Xaa₄ each independently represent any naturally occurring amino acid or

15

bc) an amino acid sequence (His)_n (Seq. Id. No. 4), wherein n stands for an integer from 4 to 6, wherein the C-terminus of aa), ab) or ac) is bound to the N-terminus of one of the sequences Seq. Id. No. 2, Seq. Id. No. 3 or Seq. Id. No. 4 via a peptide bond,

20

for binding a radioisotope, e.g. a radioisotope of Technetium or Rhenium.

The antibody fragment L19 is defined by the following sequence (Seq. Id. No. 1):

(VH)

25

```
EVQLLES GGG LVQPGGSLRL SCAASGFTFS
SFSMSWVRQA PGKGLEWVSS ISGSSGTTY
ADSVKGRFTI SRDNSKNTLY LQMNSLRAED
TAVYYCAKPF PYFDYWGGGT LVTVSS
```

(Linker)

30

```
GDGSSGGSGG ASTG
```

(VL)

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EIVLTQSPGT LSLSPGERAT LSCRASQSVS
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and/or substitution, and which has the same function as the peptide according to Seq. Id. No. 1, is defined as a peptide that binds to the ED-B domain of fibronectin with a dissociation constant K_d that is in the subnanomolar range (i.e. less than 10^{-9}), measured with a BIAcore (see
5 WO99/58570, Example 2 and Table 2).

Preferred amino acid sequences Xaa₁-Xaa₂-Xaa₃-Cys (Seq. Id. No. 2) are the sequences Gly-Gly-Gly-Cys (Seq. Id. No. 5) and Gly-Cys-Gly-Cys (Seq. Id. No. 6). Most preferred is the sequence Gly-Gly-Gly-Cys (Seq. Id. No. 5).

Preferred amino acid sequences Xaa₁-Xaa₂-Xaa₃-Cys-Xaa₄ (Seq. Id. No. 3) are the sequences Gly-Gly-Gly-Cys-Ala (Seq. Id. No. 7) and Gly-Cys-Gly-Cys-Ala (Seq. Id. No. 8). Most preferred is the sequence Gly-Gly-Gly-Cys-Ala (Seq. Id. No. 7).

In compounds comprising an amino acid sequence (His)_n (Seq. Id. No. 4), those compounds wherein n stands for the integer 6 are preferred.

Preferred radioisotopes of Technetium or Rhenium are the isotopes ^{94m}Tc,
20 ^{99m}Tc, ¹⁸⁶Re and ¹⁸⁸Re. Most preferred is the radioisotope ^{99m}Tc.

Detailed Description of the Invention

The single-chain antibody fragment L19 (Seq. Id. No. 1) was previously
25 labeled with ¹²⁵I to investigate the biodistribution of this compound in tumour-bearing mice (Tarli et al., Blood, Vol. 94, No. 1 (1999), p. 192 – 198). The results show that a selective targeting of tumoural blood vessels in vivo may be accomplished. Surprisingly however, it was found that the pharmacokinetic properties of the single-chain antibody fragment L19 may
30 be substantially improved when it is conjugated to a peptide (a), (b) or (c) and labelled with radioisotopes of Technetium or Rhenium. The isotope ^{99m}Tc is the radiolabel of choice for routine clinical SPECT due to its

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radiochemical properties (easily available through a $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator, emits single gamma-photons of 140 KeV, has high photon flux, and decays with a half-life of 6 hours) and due to its cost-effectiveness. For therapeutic applications, labeling with the chemically analogous isotopes ^{186}Re and ^{188}Re is especially preferred (Hsieh, B.T., et al., Nucl. Med. Biol., 1999, 26(8), 967-972; 973-976, Zamora, P.O., et al., Anticancer Res., 1997, 17(3B), 1803-1838).

The peptides of the present invention are derivatives of the recombinant scFv antibody L19 (Seq. Id. No. 1) against the extracellular ED-B domain of fibronectin and were produced via genetic engineering according to Fig. 1. The following peptides were produced:

L19 (Seq. Id. No. 1)

L19His:

```

1      EVQLLESGGG LVQPGGSLRL SCAASGFTFS SFSMSWVRQA
      PGKGLEWVSS
20      51      ISGSSGTTY' ADSVKGRFTI SRDNSKNTLY LQMNSLRAED
      TAVYYCAKPF
      101      PYFDYWGGQT LVTVSSGDGS SGGSGGASEI VLTQSPGTLN
      LSPGERATLS
      151      CRASQSVSSS FLAWYQQKPG QAPRLIIYYA SSRATGIPDR
25      FSGSGSGTDF
      201      TLTISRLEPE DFAVYYCQQT GRIPPTFGQG TKVEIKAAAL
      EHHHHHHH

```

(Seq. Id. No. 9)

AP38:

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1 EVQLLESGGG LVQPGGSLRL SCAASGFTFS SFSMSWVRQA
PGKGLEWVSS

51 ISGSSGTTY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED
TAVYYCAKPF

5 101 PYFDYWGGT LVTSSGDGS SGGSGGASEI VLTQSPGTL
LSPGERATLS

151 CRASQSVSSS FLAWYQQKPG QAPRLIYYA SSRATGIPDR
FSGSGSGTDF

201 TLTISRLEPE DFAVYYCQQT GRIPPTFGQG TKVEIKGGGC

10

(Seq. Id. No. 10)

AP39:

15 1 EVQLLESGGG LVQPGGSLRL SCAASGFTFS SFSMSWVRQA
PGKGLEWVSS

51 ISGSSGTTY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED
TAVYYCAKPF

101 PYFDYWGGT LVTSSGDGS SGGSGGASEI VLTQSPGTL
LSPGERATLS

20 151 CRASQSVSSS FLAWYQQKPG QAPRLIYYA SSRATGIPDR
FSGSGSGTDF

201 TLTISRLEPE DFAVYYCQQT GRIPPTFGQG TKVEIKGGGC A

25 (Seq. Id. No. 11)

L19-GlyCysGlyCys:

30 1 EVQLLESGGG LVQPGGSLRL SCAASGFTFS SFSMSWVRQA
PGKGLEWVSS

51 ISGSSGTTY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED
TAVYYCAKPF

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101 PYFDYWGQGT LVTVSSGDGS SGGSGGASEI VLTQSPGTLT
LSPGERATLS
151 CRASQSVSSS FLAWYQQKPG QAPRLIIYYA SSRATGIPDR
FSGSGSGTDF
5 201 TLTISRLEPE DFAVYYCQQT GRIPPTFGQG TKVEIKGCGC

(Seq. Id. No. 12)

L19-GlyCysGlyCysAla:

10 1 EVQLLESGGG LVQPGGSLRL SCAASGFTFS SFSMSWVRQA
PGKGLEWVSS
51 ISGSSGTTY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED
TAVYYCAKPF
15 101 PYFDYWGQGT LVTVSSGDGS SGGSGGASEI VLTQSPGTLT
LSPGERATLS
151 CRASQSVSSS FLAWYQQKPG QAPRLIIYYA SSRATGIPDR
FSGSGSGTDF
201 TLTISRLEPE DFAVYYCQQT GRIPPTFGQG TKVEIKGCGC A
20

(Seq. Id. No. 13)

The production of the peptides is described in detail in the following examples (see „Experimental“).

25 The antibody fragment L19 was originally produced by expression in *E. coli* (see WO 99/58570). However, for the large-scale production of scFv antibody fragments, this expression system was found to be unsatisfying. Another expression system, a yeast expression system, particularly a
30 *Pichia pastoris* expression system, was tested. The present inventors found that yeast, e.g. *Pichia pastoris* is generally capable for expression of a highly bioactive antibody fragment, e.g. the fragment AP39, but a high

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yield expression with up to 250 mg antibody fragment per liter culture, which is necessary for an economical production of a biopharmaceutical, could only be reached by a constitutive expression vector (e.g. pGAP), and not with a methanol inducible vector (e.g. pPIC9K). An additional
5 advantage of this constitutive expression system is its simplified and robust fermentation procedures compared to an inducible yeast expression. Unexpectedly, the present inventors found that a proper signal sequence processing of the antibody fragment, e.g. the fragment AP39 was observed only when an expression cassette was used in which the N-
10 terminus of the fragment was directly fused to the Kex2-cleavage site from the alpha-signal sequence.

The peptides are suitable for diagnostic and therapeutic applications, particularly for the diagnosis and therapy of invasive tumours and tumour
15 metastases. Preferred diagnostic applications are SPECT (Single Photon Emission Computed Tomography) and PET (Positron Emission Tomography).

The peptides described above are particularly well suited for labeling
20 radioisotopes as described above, e.g. radioisotopes of Technetium and Rhenium, preferably the radionuclides ^{94m}Tc , ^{99m}Tc , ^{186}Re , and ^{188}Re . For labeling the peptides, the peptides are first reduced with an appropriate reducing agent like e.g. stannous chloride or Tris(2-carboxyethyl)phosphine (TCEP). The resulting reduced peptides exhibit SH-groups that can react
25 with ^{99m}Tc generator eluate or ^{188}Re generator eluate and stannous chloride to the compounds of the present invention (for details, see the experimental examples below). Indirect labeling is performed by pre-conjugating a chelating ligand and subsequent complexation of radioisotopes, such as Indium, Yttrium, lanthanides etc. The chelating
30 ligand is preferably derived from ethylene diamine tetraacetic acid (EDTA), diethylene triamine pentaacetic acid (DTPA), cyclohexyl 1,2-diamine tetraacetic acid (CDTA), ethyleneglycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-

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diacetic acid (HBED), triethylene tetraamine hexaacetic acid (TTHA), 1,4,7,10-tetraazacyclododecane-N,N',N'''-tetraacetic acid (DOTA), 1,4,7-triazacyclononane-N,N',N'''-triacetic acid (NOTA), and 1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid (TETA), to either
5 amine or thiol groups of the peptide compounds. The chelating ligands possess a suitable coupling group e.g. active esters, maleimides, thiocarbamates or α -halogenated acetamide moieties. For conjugating chelating ligands to amine groups e.g. ϵ -NH₂-groups of lysine residues previous reduction of the peptide compounds is not required. The
10 radiolabeled peptides are suitable for radio-diagnostic and radio-therapeutic applications.

The resulting radiolabeled peptides show unexpected advantages in animal experiments. For example, excretion of a labeled peptide, e.g. ^{99m}Tc-labeled
15 AP39 (Seq. Id. No. 11) in nude mice occurs to 70% or more, e.g. 80.63% within 24 hours via the kidneys, whereas for L19 (Seq. Id. No. 1) labeled with ¹²⁵I, excretion in nude mice occurred only to 67.79% via the kidneys within 24 hours. The tumour to blood ratio of a labeled peptide, e.g. ^{99m}Tc-labeled AP39 is 5:1 or more, preferably 8:1 or more, e.g. about 10 : 1
20 after 5 hours, whereas for L19 labeled with ¹²⁵I, this ratio is only about 3 : 1. This is an unexpected behaviour also compared to other scFv antibodies labeled with ^{99m}Tc which often show less favourable biodistribution characteristics. For example, Verhaar et al., J. Nuc. Med., Vol. 37(5), pp. 868 – 872, 1996, report a ^{99m}Tc-labeled scFv antibody that
25 shows a tumour to blood ratio of only 4 : 1 after 24 hours, and a kidney accumulation of 9% after 24 hours, which is very high compared to the values of the peptides described in the present invention, e.g. 1.3% for ^{99m}Tc-labeled AP39 (see example 13 below).

30 Further, the in vivo stability of the labeled peptides of the invention, e.g. ^{99m}Tc-labeled AP39 is much higher compared to the in vivo stability of L19 labeled with ¹²⁵I. The present inventors found that 2 hours after injection of

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a peptide, e.g. ^{99m}Tc -labeled AP39 only 10% or less, e.g. 3% of radioactivity within the serum was due to a metabolite, whereas 2 hours after injection of L19 labeled with ^{125}I , 49% of the radioactivity in the serum was due to metabolites, which may be free iodine. The improved *in vivo* stability of the peptides, e.g. ^{99m}Tc -labeled AP39 is also reflected by a prolonged preservation of its binding ability to the target ED-B. The present inventors found that 2 hours after injection of the peptide, e.g. ^{99m}Tc -labeled AP39, 50% or more, e.g. 74% of radioactivity within the serum was able to bind ED-B, whereas 2 hours after injection of ^{125}I -labeled L19, only 27% of radioactivity within the serum could bind to ED-B. The compounds of this invention are also showing high tumour accumulation. For example, $\text{Tc-}^{99m}\text{-AP39}$ and $\text{In-}^{111}\text{-MX-DTPA-}\epsilon\text{-HN(Lys)-AP39}$ displayed high tumour accumulation of 10.7 (Tc-^{99m}) or 12.9 (In-^{111}) % injected dose per gram (ID/g) at 1 hour post injection (p.i.). Thus, tumor uptake is significantly higher compared to other known In-^{111} or Tc-^{99m} labeled antibody fragments (e.g. Kobayashi et al., J. Nuc. Med., Vol. 41(4), pp. 755 – 762, 2000; Verhaar et al., J. Nuc. Med., Vol. 37(5), pp. 868 – 872, 1996).

The compounds are suitable for diagnostic and therapeutic applications. They are preferably applied to the patient by parenteral administration, more preferably by intravenous injection. The human dose is preferably in the range of 0.1 to 1 mg per patient for radiodiagnostic applications, and 0.1 to 100 mg per patient for radiotherapeutic applications.

The methods for making and labeling the compounds of the present invention are more fully illustrated in the following examples. These examples are shown by way of illustration and not by way of limitation.

Experimental

Example 1: Production of L19 derivatives

- 5 A recombinant antibody (scFv L19, short name L19) against the extra domain B (ED-B) of a splice variant of fibronectin formed the starting material. scFv L19 had been isolated by means of phage display selection from a synthetic human antibody repertoire (Neri et al., 1997, Nature Biotechnol. 15: 1271; Pini et al., 1998, J. Biol. Chem. 273: 21769). This
- 10 recombinant antibody fragment is in the form of a so-called single chain antibody fragment (scFv) and consists of a VH and VL region connected by a linker sequence (see Seq. Id. No. 1). This scFv L19 has exceptionally high affinity for ED B (K_d : 5.4×10^{-11} M).
- 15 Various derivatives of L19 were produced by genetic manipulation (see Fig. 1). To modify L19, the scFv encoding DNA was amplified by PCR (polymerase chain reaction) using primers which coded for the additional sequences, and cloned into expression vectors.
- 20 L19 derivatives:
- | | |
|----------|---|
| L19: | without additional terminal modifications |
| L19 His: | C-terminal His ₆ domain (His tag), for Ni chelate chromatography and for binding radioisotopes |
| 25 AP38: | C-terminal GlyGlyGlyCys domain for binding (via Cys) substances which can be employed in therapy and diagnosis (e.g. radioisotopes) |
| AP39: | C-terminal GlyGlyGlyCysAla domain for binding (via Cys) substances which can be employed in |
| 30 | therapy and diagnosis (e.g. radioisotopes) |

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- L19-GlyCysGlyCys: C-terminal GlyCysGlyCys domain for binding (via Cys) substances which can be employed in therapy and diagnosis (e.g. radioisotopes)
- L19-GlyCysGlyCysAla: C-terminal GlyCysGlyCysAla domain for binding (via Cys) substances which can be employed in therapy and diagnosis (e.g. radioisotopes)

Recombinant production of L19 derivatives

- The L19 derivatives described were produced in prokaryotic and eukaryotic expression systems.

a) L19 production in E. coli

- The DNA sequences encoding various L19 derivatives (AP38, AP39, L19-GlyCysGlyCys, L19-GlyCysGlyCysAla, L19, L19His) were cloned into a prokaryotic expression vector (pDN5, Pini et al., 1997, J. Immunol. Methods 206: 171, Pini et al., 1998, J. Biol. Chem. 273: 21769; pET, Novagen) with IPTG-inducible promoter and ampicillin resistance marker. In order to make secretion of the recombinant protein into the periplasm possible, this vector was used to produce an expression cassette in which the N terminus of scFv is fused to a Pel B signal sequence. It was possible to establish stable producer strains by transforming E. coli (TG1, BL21DE3 and HB2151) with this expression vector, followed by ampicillin selection. To produce scFv, these strains were cultivated in the presence of 1% glucose in the growth phase (37°C) in order to repress the promoter. Expression of scFv in the cultures was induced by adding IPTG and incubating at 30°C for up to 16 h. Soluble and antigen-binding scFv material could be isolated from the complete extract of the E. coli strains, from the periplasm fraction or, which proved to be particularly efficient in relation to purification and yield, from the culture supernatant. Production

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took place in shaken flasks and in fermenters with a culture volume of up to 10 litres.

b) Production of L19 derivatives in Pichia pastoris

- 5 L19His, AP38, AP39, L19-GlyCysGlyCys and L19-GlyCysGlyCysAla-
encoding DNA sequences were amplified by PCR and cloned into *E. coli*
and into the expression vectors pPIC9K and pGAP (Invitrogen) for
production in the yeast *Pichia pastoris*. For expression of heterologous
genes, pPIC9K contains a methanol-inducible promoter (AOX1), and pGAP
10 contains the constitutive promoter of the GAPDH enzyme. In addition,
these vectors contain respectively a geneticin resistance gene and a zeocin
resistance gene for selection/amplification of the foreign gene and a signal
sequence (from yeast α factor) for expression and secretion of the
recombinant product. The AP39 expression cassette used codes for a
15 fusion protein (α factor signal + L19 derivatives) which contains for signal
sequence elimination only a Kex2 cleavage site and not the other cleavage
sites of natural α factor processing. Stable transfected PP clones were
established by electroporation of the linearized vectors into *Pichia pastoris*
strains (e.g. pPIC9K-AP39 into strain GS115, pGAP-AP39 into strain X33)
20 and subsequent geneticin or zeocin selection. It was possible to use these
clones to produce the said L19 derivatives as soluble secretory protein.
The clones were cultivated at 30°C in BMGY medium or basal mineral
medium. With clones based on pPIC, methanol was added for promoter
induction during the expression phase. The recombinant product had a
25 correctly processed terminus and high antigen-binding activity. The yields
which could be achieved (unpurified, bioactive product/litre of culture
supernatant) were, depending on the culturing conditions and process
control: e.g. pPIC9K-AP39/GS115 (shaken flask 5 mg/l, fermenter 10-
15 mg/l); pGAP-AP39/X33 (shaken flask 30-40 mg/l, fermenter 100-
30 250 mg/l).

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The L19 derivatives were purified from the *Pichia pastoris* or *E. coli* culture supernatant by use of affinity chromatography (rProtein A, Streamline Pharmacia or ED B antigen column) with subsequent size exclusion chromatography. The purified AP39 fraction, which was employed for further processing, had a homodimer structure (with subunits covalently linked for the most part) and high antigen-binding activity.

Example 2a

10 **Synthesis of reduced AP38 [Reduced L19-(Gly)₃-Cys-OH]**

To a solution of 240 μ g (4.29 nmol) S-S-dimeric AP38 in 156 μ l PBS (phosphate buffered saline)/10% glycerine were added 50 μ l TCEP-solution (14.34 mg TCEP x HCl/5 ml aqueous Na₂HPO₄, 0.1 M, pH = 7.4). The reaction mixture was gently shaken for 1 h at room temperature. SH-monomeric AP38 was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS). SDS-PAGE analysis of the isolated product proofed the quantitative transformation of S-S-dimeric AP38 to SH-monomeric AP38.

20 Yield: 79.4 μ g/220 μ l PBS (33.1%).

Example 2b

Synthesis of Tc-99m-AP38 [Tc-99m-L19-(Gly)₃-Cys-OH]

25

2.37 mg disodium-L-tartrate were placed in a vial followed by addition of 79.4 μ g reduced AP38 in 220 μ l PBS and the solution was diluted with 100 μ l aqueous Na₂HPO₄-buffer (1 M, pH = 10.5). 50 μ l Tc-99m generator

- 20 -

eluate (24 h) and 10 μ l SnCl_2 -solution (5 mg SnCl_2 /1 ml 0.1 M HCl) were added. The reaction mixture was shaken for 0.5 h at 37°C. Tc-99m-labeled AP38 was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

- 5 Radiochemical yield: 39.7%.
Radiochemical purity: 92.5% (SDS-PAGE).
Specific activity: 17.7 MBq/nmol.
Immunoreactivity: 88.7%

10 Example 3a

Synthesis of reduced AP39 [Reduced L19-(Gly)₃-Cys-Ala-OH]

- To a solution of 240 μ g (4.29 nmol) S-S-dimeric AP39 in 135 μ l PBS/10%
15 glycerine were added 50 μ l TCEP-solution (14.34 mg TCEP x HCl/5 ml
aqueous Na_2HPO_4 , 0.1 M, pH = 7.4). The reaction mixture was gently
shaken for 1 h at room temperature. SH-monomeric AP39 was purified by
gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS). SDS-
PAGE analysis of the isolated product proofed the quantitative
20 transformation of S-S-dimeric AP39 to SH-monomeric AP39.

Yield: 135.9 μ g/180 μ l PBS (56.2%).

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Example 3b**Synthesis of Tc-99m-AP39 [Tc-99m-L19-(Gly)₃-Cys-Ala-OH]**

- 5 4.2 mg disodium-L-tartrate were placed in a vial followed by addition of 135.9 μ g reduced AP39 in 180 μ l PBS and the solution was diluted with 100 μ l aqueous Na₂HPO₄-buffer (1 M, pH = 10.5). 100 μ l Tc-99m generator eluate (24 h) and 10 μ l SnCl₂-solution (5 mg SnCl₂/1 ml 0.1 M HCl) were added. The reaction mixture was shaken for 0.5 h at 37°C. Tc-
10 99m-labeled AP39 was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield: 50.1%.

Radiochemical purity: 91.5% (SDS-PAGE).

Specific activity: 21.4 MBq/nmol.

- 15 Immunoreactivity: 96.4%

Example 4**Synthesis of Re-188-AP38 [Re-188-L19-(Gly)₃-Cys-OH]**

20

- 2.37 mg disodium-L-tartrate were placed in a vial followed by addition of 112 μ g reduced AP38 in 310 μ l PBS and the solution was diluted with 100 μ l aqueous Na₂HPO₄-buffer (1 M, pH = 10.5). 100 μ l Re-188 generator eluate and 50 μ l SnCl₂-solution (5 mg SnCl₂/1 ml 0.1 M HCl) were added.
25 The reaction mixture was shaken for 1.5 h at 37°C. Re-188-labeled AP38 was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield: 28.3%.

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Radiochemical purity: 91.1% (SDS-PAGE).

Specific activity: 15.3 MBq/nmol.

Immunoreactivity: 89.9%

5 **Example 5**

Synthesis of Re-188-AP39 [Re-188-L19-(Gly)₃-Cys-Ala-OH]

2.37 mg disodium-L-tartrate were placed in a vial followed by addition of
112 µg reduced AP39 in 303 µl PBS and the solution was diluted with 100
µl aqueous Na₂HPO₄-buffer (1 M, pH = 10.5). 100 µl Re-188 generator
eluate and 50 µl SnCl₂-solution (5 mg SnCl₂/1 ml 0.1 M HCl) were added.
The reaction mixture was shaken for 1.5 h at 37°C. Re-188-labeled AP39
was purified by gel-chromatography using a NAP-5 column (Amersham,
Eluent: PBS).

Radiochemical yield: 33.5%.

Radiochemical purity: 92.3% (SDS-PAGE).

Specific activity: 18.5 MBq/nmol.

Immunoreactivity: 92.5%

Example 6a

Synthesis of reduced L19-Gly-Cys-Gly-Cys-OH

To a solution of 240 µg (4.29 nmol) S-S-dimeric L19-Gly-Cys-Gly-Cys-OH
in 160 µl PBS/10% glycerine were added 75 µl TCEP-solution (14.34 mg
TCEP x HCl/5 ml aqueous Na₂HPO₄, 0.1 M, pH = 7.4). The reaction

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mixture was gently shaken for 1 h at room temperature. SH-monomeric L19-Gly-Cys-Gly-Cys-OH was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS). SDS-PAGE analysis of the isolated product proofed the quantitative transformation of S-S-dimeric L19-Gly-Cys-Gly-Cys-OH to SH-monomeric L19-Gly-Cys-Gly-Cys-OH.

Yield: 80.4 μg /210 μl PBS (33.5%).

Example 6b

Synthesis of Tc-99m-L19-Gly-Cys-Gly-Cys-OH

2.37 mg disodium-L-tartrate were placed in a vial followed by addition of 80.4 μg reduced L19-Gly-Cys-Gly-Cys-OH in 210 μl PBS and the solution was diluted with 100 μl aqueous Na_2HPO_4 -buffer (1 M, pH = 10.5). 50 μl Tc-99m generator eluate (24 h) and 10 μl SnCl_2 -solution (5 mg SnCl_2 /1 ml 0.1 M HCl) were added. The reaction mixture was shaken for 0.5 h at 37°C. Tc-99m-labeled L19-Gly-Cys-Gly-Cys-OH was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield: 37.7%.

Radiochemical purity: 91.5% (SDS-PAGE).

Specific activity: 19.7 MBq/nmol.

Immunoreactivity: 89.7%

Example 7a**Synthesis of reduced L19-Gly-Cys-Gly-Cys-Ala-OH**

- 5 To a solution of 240 μg (4.29 nmol) S-S-dimeric L19-Gly-Cys-Gly-Cys-Ala-OH in 155 μl PBS/10% glycerine were added 75 μl TCEP-solution (14.34 mg TCEP \times HCl/5 ml aqueous Na_2HPO_4 , 0.1 M, pH = 7.4). The reaction mixture was gently shaken for 1 h at room temperature. SH-monomeric L19-Gly-Cys-Gly-Cys-Ala-OH was purified by gel-chromatography using a
- 10 NAP-5 column (Amersham, Eluent: PBS). SDS-PAGE analysis of the isolated product proofed the quantitative transformation of S-S-dimeric L19-Gly-Cys-Gly-Cys-Ala-OH to SH-monomeric L19-Gly-Cys-Gly-Cys-Ala-OH.

Yield: 81.2 μg /215 μl PBS (33.8%).

15

Example 7b**Synthesis of Tc-99m-L19-Gly-Cys-Gly-Cys-Ala-OH**

- 20 2.37 mg disodium-L-tartrate were placed in a vial followed by addition of 81.2 μg reduced L19-Gly-Cys-Gly-Cys-Ala-OH in 215 μl PBS and the solution was diluted with 100 μl aqueous Na_2HPO_4 -buffer (1 M, pH = 10.5). 50 μl Tc-99m generator eluate (24 h) and 10 μl SnCl_2 -solution (5 mg SnCl_2 /1 ml 0.1 M HCl) were added. The reaction mixture was shaken for
- 25 0.5 h at 37°C. Tc-99m-labeled L19-Gly-Cys-Gly-Cys-Ala-OH was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield: 35.6%.

Radiochemical purity: 93.5% (SDS-PAGE).

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Specific activity: 19.1 MBq/nmol.

Immunoreactivity: 88.7%

Example 8a

5

Synthesis of reduced AP39 for specific conjugation of EDTA, CDTA, TETA, DTPA, TTTHA, HBED, DOTA, NOTA, and DO3A type chelators to the Cysteine-SH group

- 10 50 μ l TCEP-solution (14.34mg TCEP \times HCl/5ml aqueous Na₂HPO₄, 0.1M, pH = 7.4) were added to a solution of 400 μ g (7.1 nmol) AP39 in 450 μ l PBS. The reaction mixture was gently shaken for 1h at 37 °C. Reduced AP39 was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: sodium acetate buffer, 0.1M, pH 5.0). SDS-PAGE analysis of the
- 15 isolated product proofed the complete transformation of AP39 into reduced AP39.

Yield: 140 μ g/200 μ l (35%).

Example 8b

20

Synthesis of MX-DTPA-Maleimide (1,4,7-tris(2-(N-maleimido ethylene *p*-amino)benzyl-1,7-bis(carboxymethyl)-4-carboxymethyl 6-methyl heptane)

- 512 mg (1 mmol) of {[3-(4-Amino-phenyl)-2-(bis-carboxymethyl-amino)-propyl]-[2-(bis-carboxymethyl-amino)-propyl]-amino}-acetic acid (Macrocylics Inc. Dallas, TX, U.S.A.) and 707 mg (7 mmol) triethylamine were dissolved in 3 ml dry DMF. 400 mg (1,5 mmol) of 3-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)-propionic acid 2,5-dioxo-pyrrolidin-1-yl ester (Aldrich)
- 25

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- in 1 ml dry DMF were added dropwisely. The solution was stirred for 5 h at 50° C. 30 ml of diethylether were added slowly. The reaktion mixture was stirred for further 30 min. The principate was collected by filtering. The crude product was purified by RP-HPLC (acetonitrile- : water- : trifluoracetic acid / 3 : 96,9 : 0,1 → 99,9 : 0 : 0,1). Yield: 61% (405 mg, 0,61 mmol). MS-ESI: 664 = M⁺ + 1.

Example 8c

10 **Synthesis of In-111-MX-DTPA-Maleimide-S(Cys)-AP39-R**

(R = reduced)

- 140 µg (5 nmol) AP39-R in 200 µl of sodium acetate buffer (0.1M, pH 5) were reacted with 50µl of dissolved 1,4,7-triaza-2-(N-maleimido ethylene *p*-amino)benzyl-1,7-bis(carboxymethyl)-4-carboxymethyl-6-methylheptane (0,25mg DTPA-Maleimide in 500µl sodium acetate buffer 0.1M pH 5) for 3 h at 37°C. The reaction mixture was dialyzed 2 x 1 h with 200ml of sodium acetate buffer (0.1M, pH 6) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.).

20

80 µl [In-111]InCl₃ solution (HCl, 1N, 40 MBq, Amersham Inc.) were added and the reaction mixture was heated at 37°C for 30 min. In-111 labeled DTPA-Maleimide-S(Cys)-AP39-R was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

- 25 Radiochemical yield: 54 %.
- Radiochemical purity: 94 % (SDS-PAGE).
- Specific activity: 6.2 MBq/nmol.
- Immunoreactivity: 86 %

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Example 9**Synthesis of In-111-MX-DTPA- ϵ -HN(Lys)-AP39**

- 200 μ g (3.6 nmol) non-reduced AP39 in 111 μ l PBS were diluted with 300 μ l of sodium borate buffer (0.1M, pH 8.5) and dialyzed 2 x 1 h with 200ml of sodium borate buffer (0.1M, pH 8.5) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.). 50 μ l of 1,4,7-triaza-2-(p-isothiocyanato)benzyl-1,7-bis(carboxymethyl)-4-carboxymethyl-6-methyl heptane (MX-DTPA) solution (0.33 mg MX-DTPA dissolved in 500 μ l of sodium borate buffer, 0.1M, pH 8.5) were added and the reaction mixture was heated for 3 h at 37 °C. The reaction mixture was dialyzed 2 x 1 h and 1 x 17 h (over night) with 200 ml of sodium acetate buffer (0.1M, pH 6.0) each, employing the Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.).

- 80 μ l [In-111]InCl₃ solution (HCl, 1N, 40 MBq, Amersham Inc.) were added and the reaction mixture was heated at 37 °C for 30 min. In-111 labeled MX-DTPA- ϵ -HN(Lys)-AP39 was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield:	70 %.
Radiochemical purity:	85 % (SDS-PAGE).
Specific activity:	7.6 MBq/nmol.
Immunoreactivity:	74 %

25

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Example 10**Synthesis of In-111 -DGT A-C-Benzyl-p-NCS - ϵ -HN(Lys)-AP39**

- 5 200 μ g (3.6 nmol) non-reduced AP39 in 114 μ l PBS were diluted with 300 μ l of sodium borate buffer (0.1M, pH 8.5) and dialyzed 2 x 1 h with 200ml of sodium borate buffer (0.1M, pH 8.5) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.). 50 μ l of 1,4,7,10-tetraaza-2-(p-isothiocyanato)benzyl cyclododecane-1,4,7,10-tetraacetic acid (benzyl-p-SCN-DOTA, Macrocyclics Inc., Dallas TX, U.S.A.) solution (1.5 mg benzyl-p-SCN-DOTA dissolved in 5 ml of sodium borate buffer, 0.1M, pH 8.5) were added to the solution and the reaction mixture was heated for 3 h at 37 °C. The reaction mixture was dialyzed 2 x 1 h and 1 x 17 h (over night) with 200 ml of sodium acetate buffer (0.1M, pH 6.0) each, employing the
- 15 Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.).

- 80 μ l [In-111]InCl₃ solution (HCl, 1N, 40 MBq, Amersham Inc.) were added and the reaction mixture was heated at 37 °C for 30 min. In-111 labeled DOTA-C-Benzyl-p-NCS- ϵ -HN(Lys)-AP39 was purified by gel-
- 20 chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield:	74 %.
Radiochemical purity:	94 % (SDS-PAGE).
Specific activity:	12.3 MBq/nmol.
Immunoreactivity:	73 %

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Example 11**Synthesis of Y-88-MX-DTPA- ϵ -HN(Lys)-AP39**

- 5 200 μ g (3.6 nmol) non-reduced AP39 in 115 μ l PBS were diluted with 300 μ l of sodium borate buffer (0.1M, pH 8.5) and dialyzed 2 x 1 h with 200ml of sodium borate buffer (0.1M, pH 8.5) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.). 50 μ l of MX-DTPA solution (0.33 mg MX-DTPA dissolved in 500 μ l of sodium borate buffer, 0.1M, pH
- 10 8.5) were added and the reaction mixture was heated for 3 h at 37 °C. The reaction mixture was dialyzed 2 x 1 h and 1 x 17 h (over night) with 200 ml of sodium acetate buffer (0.1M, pH 6.0) each, employing the Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.).
- 15 100 μ l [Y-88]YCl₃ solution (HCl, 1N, 75 MBq, Oak Ridge National Lab.) were added and the reaction mixture was heated at 37 °C for 30 min. Y-88 labeled MX-DTPA- ϵ -HN(Lys)-AP39 was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).
- Radiochemical yield: 65 %.
- 20 Radiochemical purity: 93 % (SDS-PAGE).
- Specific activity: 10.2 MBq/nmol.
- Immunoreactivity: 72 %

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Example 12**Synthesis of Lu-177 -DOTA-C-Benzyl-p-NCS- ϵ -HN(Lys)-AP3**

- 5 200 μ g (3.6 nmol) non-reduced AP39 in 110 μ l PBS were diluted with 300 μ l of sodium borate buffer (0.1M, pH 8.5) and dialyzed 2 x 1 h with 200ml of sodium borate buffer (0.1M, pH 8.5) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.). 50 μ l of benzyl-p-SCN-DOTA solution (1.5 mg dissolved in 5 ml of sodium borate buffer, 0.1M, pH 8.5)
- 10 were added and the reaction mixture was heated for 3 h at 37 °C. The reaction mixture was dialyzed 2 x 1 h and 1 x 17 h (over night) with 200 ml of sodium acetate buffer (0.1M, pH 6.0) each, employing the Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.).
- 15 200 μ l [Lu-177]LuCl₃ solution (HCl, 1N, 80 MBq, NRH-Petten, Netherlands) were added and the reaction mixture was heated at 37 °C for 30 min. Lu-177 labeled DOTA-C-Benzyl-p-NCS- ϵ -HN(Lys)-AP39 was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).
- Radiochemical yield: 74 %.
- 20 Radiochemical purity: 95 % (SDS-PAGE).
- Specific activity: 19 MBq/nmol.
- Immunoreactivity: 71 %

Example 13

Organ distribution and excretion of Tc-99m-AP39, expressed in *Pichia pastoris*, after a single i.v. injection into tumour-bearing nude mice

5

The substance of the invention is injected intravenously in a dose of about 74 kBq into F9 (teratocarcinoma)-bearing animals (bodyweight about 25 g). The radioactivity concentration in various organs, and the radioactivity in the excreta are measured using a γ counter at various times after administration of the substance. In addition, the tumour to blood ratio is found at various times on the basis of the concentration of the substance of the invention in tumour and blood.

10

The biodistribution of Tc-99m-AP39 in F9 (teratocarcinoma)-bearing nude mice (mean \pm SD, n=3) is shown in Table 2:

15

20

25

	% of dose / g of tissue	% of dose / g of tissue	% of dose / g of tissue
	1 h p.i.	5 h p.i.	24 h p.i.
Spleen	1.97 \pm 0.018	0.53 \pm 0.07	0.31 \pm 0.08
Liver	1.91 \pm 0.046	0.77 \pm 0.07	0.26 \pm 0.01
Kidney	19.21 \pm 0.70	4.35 \pm 0.082	1.32 \pm 0.10
Lung	3.43 \pm 1.01	1.41 \pm 0.032	0.96 \pm 0.23
Stomach without contents	1.55 \pm 0.043	1.35 \pm 0.22	0.48 \pm 0.10

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Intestine with contents	1.42 \pm 0.10	1.26 \pm 0.34	0.29 \pm 0.05
Tumour	10.72 \pm 3.21	5.13 \pm 1.45	3.48 \pm 1.28
Blood	3.03 \pm 0.32	0.57 \pm 0.11	0.11 \pm 0.01

Table 2

The excretion of Tc-99m-AP39 in F9 (teratocarcinoma)-bearing nude mice (mean \pm SD, n=3) is shown in Table 3:

	% of dose
	24 h p.i.
Urine	80.63 \pm 3.33
Faeces	3.94 \pm 0.17

Table 3

The tumour to blood ratio of Tc-99m-AP39 in F9 (teratocarcinoma)-bearing nude mice (mean \pm SD, n=3) is shown in Fig. 2.

The results of this investigation show the excellent potential of the substance of the invention for accumulation in solid tumours with, at the same time, excellent excretion.

Example 14

Organ distribution of In-111-MX-DTPA- ϵ -HN(Lys)-AP39 after a single i.v. injection into tumour-bearing nude mice

The substance of the invention is injected intravenously in a dose of about 48 kBq into F9 (teratocarcinoma)-bearing animals (body weight about 25 g). The radioactivity concentration in various organs, and the radioactivity in the excreta are measured using a γ counter at various times after administration of the substance.

The biodistribution of In-111-MX-DTPA- ϵ -HN(Lys)-AP39 in F9 (teratocarcinoma)-bearing nude mice (mean \pm SD, n=3) is shown in Table 4:

	% dose / g of tissue		
	1h p.i.	3h p.i.	24h p.i.
Spleen	1.94 \pm 0.49	1.28 \pm 0.13	1.18 \pm 0.24
Liver	2.61 \pm 1.32	2.59 \pm 0.36	2.26 \pm 0.75
Lung	1.52 \pm 1.57	2.36 \pm 0.30	0.76 \pm 0.21
Stomach without contents	1.44 \pm 0.81	1.40 \pm 0.31	0.65 \pm 0.28
Intestine with contents	5.05 \pm 5.26	1.07 \pm 0.34	0.67 \pm 0.11
Tumour	12.90 \pm 4.81	7.44 \pm 1.34	4.33 \pm 0.84
Blood	5.55 \pm 1.89	1.80 \pm 0.20	0.11 \pm 0.02

Table 4

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The tumour to blood ratio of In-111-MX-DTPA- ϵ -HN(Lys)-AP39' in F9 (teratocarcinoma)-bearing nude mice (mean \pm SD, n=3) is shown in Table 5.

5

	1h p.i.	3h p.i.	24h p.i.
Tumour to blood ratio	2.76 \pm 2.00	4.16 \pm 0.75	36.36 \pm 3.78

10

Table 5

The results of this investigation show the excellent potential of the substance of the invention for accumulation in solid tumours paired with excellent biodistribution and tumor to blood ratio.

15

Example 15

Imaging of Tc-99m-AP39, expressed in Pichia pastoris, after a single i.v. injection into tumour-bearing nude mice

20

The substance of the invention is injected intravenously in a dose of about 9.25 MBq into F9 (teratocarcinoma)-bearing animals (bodyweight about 25 g). Gamma-camera imaging is carried out at various times after administration of the substance.

25

Planar scintigraphy of Tc-99m-AP39 in F9 (teratocarcinoma)-bearing nude mice is shown in Figures 3 and 4. Fig. 3 shows the scintigram 5 hours after injection of the substance, and Fig. 4 shows the scintigram 24 hours after injection of the substance.

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The result of this investigation shows the excellent potential of the substance of the invention for imaging solid tumours.

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Claims

1. A compound comprising
a peptide comprising:
- 5 aa) an antigen-binding site for the extra domain B (ED-B) of
fibronectin comprising complementarity-determining regions
HCDR3 and/or LCDR3 as shown in Table 1 or a variation
thereof that is a deletion, insertion and/or O
substitution of up to 5 amino acids for the HCDR3 region and
10 up to 6 amino acids for the LCDR3 region which has the same
function as a peptide according to Seq. Id. No. 1;
- ab) an antigen-binding site for the extra domain B(ED-B) of
fibronectin comprising complementarity-determining regions
HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 as
15 shown in Table 1 or a variation thereof that is a deletion,
insertion and/or substitution of up to 3 amino acids for the
HCDR1 region, up to 8 amino acids for the HCDR2 region, up
to 5 amino acids for the HCDR3 region, up to 6 amino acids
for the LCDR1 region, up to 4 amino acids for the LCDR2
20 region and up to 6 amino acids for the LCDR3 region; which
has the same function as a peptide according to Seq. Id. No.
1; or
- ac) a sequence according to Seq. Id. No. 1 (L19) or a variation of
Seq. Id. No. 1 that is a deletion, insertion and/or substitution of
25 up to 30 amino acids, an which has the same function as a
peptide according to Seq. Id. No. 1,
and
- ba) an amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys (Seq. Id. No. 2),
wherein Xaa₁, Xaa₂ and Xaa₃ each independently represent any
30 naturally occurring amino acid or

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- bb) an amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys-Xaa₄ (Seq. Id. No. 3), wherein Xaa₁, Xaa₂, Xaa₃, and Xaa₄ each independently represent any naturally occurring amino acid or
- bc) an amino acid sequence (His)_n (Seq. Id. No. 4), wherein n stands for an integer from 4 to 6,
- wherein the C-terminus of aa), ab) or ac) is bound to the N-terminus of one of the sequences Seq. Id. No. 2, Seq. Id. No. 3 or Seq. Id. No. 4 via a peptide bond.
2. The compound according to claim 1, wherein the amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys (Seq. Id. No. 2) is the sequence Gly-Gly-Gly-Cys (Seq. Id. No. 5) or Gly-Cys-Gly-Cys (Seq. Id. No. 6).
3. The compound according to claim 1, wherein the amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys-Xaa₄ (Seq. Id. No. 3) is the sequence Gly-Gly-Gly-Cys-Ala (Seq. Id. No. 7) or Gly-Cys-Gly-Cys-Ala (Seq. Id. No. 8).
4. The compound according to claim 1, wherein n in the amino acid sequence (His)_n (Seq. Id. No. 4) is 6.
5. The compound according to any one of claims 1-4 which is conjugated to a radioisotope.
6. The compound according to claim 6 which is conjugated to a radioisotope selected from a radioisotope of Technetium, such as ^{94m}Tc, ^{99m}Tc, Rhenium, such as ¹⁸⁶Re, ¹⁸⁸Re, or other isotopes, such as ²⁰³Pb, ⁶⁷Ga, ⁶⁸Ga, ⁴³Sc, ⁴⁴Sc, ⁴⁷Sc, ^{110m}In,

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^{114}In , ^{97}Ru , ^{62}Cu , ^{64}Cu , ^{67}Cu , ^{68}Cu , ^{86}Y , ^{88}Y , ^{90}Y , ^{121}Sn , ^{161}Tb ,
 ^{153}Sm , ^{166}Ho , ^{105}Rh , ^{177}Lu , ^{72}As and ^{18}F .

7. A compound according to claim 6, wherein the radioisotope is
5 $^{99\text{m}}\text{Tc}$ or ^{188}Re .
8. The compound according to any one of claims 1-7, wherein the peptide is in reduced form.
- 10 9. A pharmaceutical composition comprising as an active agent a compound according to any one of claims 1-8 together with physiologically acceptable adjuvants, carriers and/or diluents.
- 15 10. The composition of claim 9 which is excreted to 70% or more via the kidneys within 24 hours in mice.
11. The composition of claim 9 or 10 having a tumour to blood ratio of 5:1 or more 5 h after administration in mice.
- 20 12. The composition of any one of claims 9-11 for diagnostic applications.
13. The composition of any one of claims 9-11 for therapeutic applications.
- 25 14. Use of a peptide comprising
- aa) an antigen-binding site for the extra domain B (ED-B) of fibronectin comprising complementarity-determining regions

- 39 -

HCDR3 and/or LCDR3 as shown in Table 1 or a variation thereof that is a deletion, insertion and/or substitution of up to 5 amino acids for the HCDR3 region and up to 6 amino acids for the LCDR3 region which has the same function as a peptide according to Seq. Id. No. 1;

ab) an antigen-binding site for the extra domain B(ED-B) of fibronectin comprising complementarity-determining regions HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 as shown in Table 1 or a variation thereof that is a deletion, insertion and/or substitution of up to 3 amino acids for the HCDR1 region, up to 8 amino acids for the HCDR2 region, up to 5 amino acids for the HCDR3 region, up to 6 amino acids for the LCDR1 region, up to 4 amino acids for the LCDR2 region and up to 6 amino acids for the LCDR3 region; which has the same function as a peptide according to Seq. Id. No. 1; or

ac) a sequence according to Seq. Id. No. 1 (L19) or a variation of Seq. Id. No. 1 that is a deletion, insertion and/or substitution of up to 30 amino acids, an which has the same function as a peptide according to Seq. Id. No. 1, and

ba) an amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys (Seq. Id. No. 2), wherein Xaa₁, Xaa₂ and Xaa₃ each independently represent any naturally occurring amino acid or

bb) an amino acid sequence Xaa₁-Xaa₂-Xaa₃-Cys-Xaa₄ (Seq. Id. No. 3), wherein Xaa₁, Xaa₂, Xaa₃, and Xaa₄ each independently represent any naturally occurring amino acid or

bc) an amino acid sequence (His)_n (Seq. Id. No. 4), wherein n stands for an integer from 4 to 6,

- 40 -

wherein the C-terminus of aa), ab) or ac) is bound to the N-terminus of one of the sequences Seq. Id. No. 2, Seq. Id. No. 3 or Seq. Id. No. 4 via a peptide bond,
for binding a radioisotope.

5

15. The use according to claim 14, wherein the radioisotope is selected from a radioisotope of Technetium, such as ^{94m}Tc , ^{99m}Tc , Rhenium, such as ^{186}Re , ^{188}Re , or other isotopes, such as ^{203}Pb , ^{67}Ga , ^{68}Ga , ^{43}Sc , ^{44}Sc , ^{47}Sc , ^{110m}In , ^{111}In , ^{97}Ru , ^{62}Cu , ^{64}Cu , ^{67}Cu , ^{68}Cu , ^{86}Y , ^{88}Y , ^{90}Y , ^{121}Sn , ^{161}Tb , ^{153}Sm , ^{166}Ho , ^{105}Rh , ^{177}Lu , ^{72}As and ^{18}F .

10

15

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25

16. The use according to claim 15, wherein the radioisotope is ^{99m}Tc or ^{188}Re .
17. A process for the production of a peptide as defined in any one of claims 1-4, characterized in that the peptide is expressed in eukaryotic cells, particularly in yeast cells.
18. The process according to claim 17, wherein the eukaryotic cells are *Pichia pastoris* cells.
19. The process according to claim 17 or 18, wherein the peptide is expressed constitutively.
20. The process according to any one of claims 17-19, wherein the N-terminus of the peptide is directly fused to the Kex2-cleavage site from the α -signal sequence.

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21. A kit for the production of radiopharmaceuticals comprising a peptide as defined in any one of claims 1-8, optionally together with physiologically acceptable adjuvants.

Exemplar 1

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Fig. 1

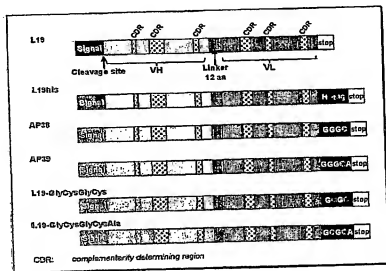
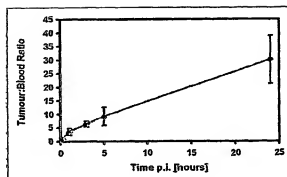
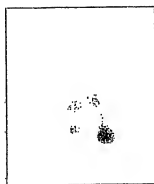
L19-Derivatives: Expression Cassettes with Protein-Encoding Regions

Fig. 2



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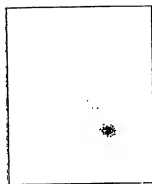
Fig. 3



5h p.i.

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Fig. 4



24h p.i.

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20 25 30

Ser Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ser Ser Ile Ser Gly Ser Ser Gly Thr Thr Tyr Tyr Ala Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Lys Pro Phe Pro Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
100 105 110

Thr Val Ser Ser Gly Asp Gly Ser Ser Gly Gly Ser Gly Gly Ala Ser
115 120 125

Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser
130 135 140

Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser
145 150 155 160

Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
165 170 175

Leu Leu Ile Tyr Tyr Ala Ser Ser Arg Ala Thr Gly Ile Pro Asp Arg
180 185 190

Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg
195 200 205

Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Thr Gly Arg
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Ile Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
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 Ser Ser Ile Ser Gly Ser Ser Gly Thr Thr Tyr Tyr Ala Asp Ser Val
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 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
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 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Lys Pro Phe Pro Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
 100 105 110
 Thr Val Ser Ser Gly Asp Gly Ser Ser Gly Gly Ser Gly Gly Ala Ser
 115 120 125
 Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly
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 Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser
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 Phe Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu
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 Ile Tyr Tyr Ala Ser Ser Arg Ala Thr Gly Ile Pro Asp Arg Phe Ser
 180 185 190
 Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg Leu Glu
 195 200 205
 Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Thr Gly Arg Ile Pro
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Ser	Ser	Ile	Ser	Gly	Ser	Ser	Gly	Thr	Thr	Tyr	Tyr	Ala	Asp	Ser	Val
	50				55					60					

Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ser	Lys	Asn	Thr	Leu	Tyr
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Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
			85						90				95		

Ala	Lys	Pro	Phe	Pro	Tyr	Phe	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	Leu	Val
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Thr	Val	Ser	Ser	Gly	Asp	Gly	Ser	Ser	Gly	Gly	Ser	Gly	Gly	Ala	Ser
		115				120						125			

Glu	Ile	Val	Leu	Thr	Gln	Ser	Pro	Gly	Thr	Leu	Ser	Leu	Ser	Pro	Gly
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Ile	Tyr	Tyr	Ala	Ser	Ser	Arg	Ala	Thr	Gly	Ile	Pro	Asp	Arg	Phe	Ser
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Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
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Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
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Ala Lys Pro Phe Pro Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
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Thr Val Ser Ser Gly Asp Gly Ser Ser Gly Gly Ser Gly Gly Ala Ser
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Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly
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Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser
 145 150 155 160

Phe Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu
 165 170 175

Ile Tyr Tyr Ala Ser Ser Arg Ala Thr Gly Ile Pro Asp Arg Phe Ser
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Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg Leu Glu
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Ser Ser Ile Ser Gly Ser Ser Gly Thr Thr Tyr Trp Ala Asp Ser Val
 50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
 65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Lys Pro Phe Pro Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
100 105 110

Thr Val Ser Ser Gly Asp Gly Ser Ser Gly Gly Ser Gly Gly Ala Ser
115 120 125

Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly
130 135 140

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser
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Phe Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu
165 170 175

Ile Tyr Tyr Ala Ser Ser Arg Ala Thr Gly Ile Pro Asp Arg Phe Ser
180 185 190

Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg Leu Glu
195 200 205

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 35 40 45
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 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Lys Pro Phe Pro Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
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 Ala

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- (30) Priority Data:
02000315.8 3 January 2002 (03.01.2002) EP
60/358,702 25 February 2002 (25.02.2002) US
- (71) Applicant (for all designated States except US): SCHERING AKTIENGESELLSCHAFT [DE/DE]; Müllerstrasse 178, 13353 Berlin (DE).
- (72) Inventors: and
(75) Inventors/Applicants (for US only): HILGER, Christoph-Stepan [DE/DE]; Langenauer Weg 24, 13503 Berlin (DE). BERNDORFF, Dietmar [DE/DE]; Büchenallee 79A, 16540 Hohen Neuendorf (DE). DINKELBORG, Ludger [DE/DE]; Ortwinstrasse 7, 13465 Berlin (DE). MOOSMAYER, Dieter [DE/DE]; Schering Aktiengesellschaft, Müllerstrasse 178, 13353 Berlin (DE). NERI, Giovanni [IT/IT]; Filogen S.r.l., Lal.izza 7, I-52100 Siena (IT).
- (81) Designated States (national): AE, AG, AI, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GI, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GI, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Declaration under Rule 4.17:**
— of inventorship (Rule 4.17(iv)) for US only
- Published:**
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24 December 2003
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CONJUGATES COMPRISING AN ANTIBODY SPECIFIC FOR THE ED-B DOMAIN OF FIBRONECTIN AND THEIR USE FOR THE DETECTION AND TREATMENT OF TUMOURS

(57) Abstract: The present invention relates to methods for diagnosis and treatment of tumours, using peptides for binding radionuclides. The peptides comprise an antigen-binding site for the extra domain B (ED-B) of fibronectin.

WO 03/055917 A3

INTERNATIONAL SEARCH REPORT

 Intern. No. of Application No
 PCT/EP 03/00009

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K19/00 A61K47/48 A61K51/10 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, SEQUENCE SEARCH, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARTY CORNELIA ET AL: "Production of functionalized single-chain Fv antibody fragments binding to the ED-B domain of the B-isoform of fibronectin in <i>Pichia pastoris</i> ." PROTEIN EXPRESSION AND PURIFICATION, vol. 21, no. 1, February 2001 (2001-02), pages 156-164, XP002242574 ISSN: 1046-5928 cited in the application page 157, left-hand column; figure 1 page 162, right-hand column -page 163, left-hand column	1,17-20
Y	--- -/-	1-21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

27 May 2003

Date of mailing of the international search report

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Deck, A

INTERNATIONAL SEARCH REPORT

Intern. Application No.

PCT/EP 03/00009

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99 58570 A (EIDGENOESS TECH HOCHSCHULE) 18 November 1999 (1999-11-18) cited in the application page 4, line 9-21 page 7, line 24 -page 8, line 21; figure 6 ---	1-21
Y	GEORGE A'J T ET AL: "RADIOMETAL LABELING OF RECOMBINANT PROTEINS BY A GENETICALLY ENGINEERED MINIMAL CHELATION SITE: TECHNETIUM-99M COORDINATION BY SINGLE-CHAIN FV ANTIBODY FUSION PROTEINS THROUGH A C-TERMINAL CYSTEINYL PEPTIDE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 92, no. 18, August 1995 (1995-08), pages 8358-8362, XP000891226 ISSN: 0027-8424 cited in the application page 8362, left-hand column ---	1-21
Y	MARIANI GIULIANO ET AL: "A pilot pharmacokinetic and immunoscintigraphic study with the technetium-99m-labeled monoclonal antibody BC-1 directed against oncofetal fibronectin in patients with brain tumors." CANCER, vol. 80, no. 12 SUPPL., 15 December 1997 (1997-12-15), pages 2484-2489, XP002242575 ISSN: 0008-543X the whole document -----	1-21

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 03/00009

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		TR 200003317 T2	23-07-2001

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